A SEROLOGICAL COMPARISON OF FIVE SPECIES OF ATLANTIC CLUPEOID FISHES

DONALD F. MAIRS[1] AND CARL J. SINDERMANN

U. S. Bureau of Commercial Fisheries Biological Laboratory, Boothbay Harbor, Maine

The potential role of serology in fishery biology has received increased attention during the past decade (Cushing, 1952, 1956; Ridgway, 1957; Ridgway, Cushing and Durall, 1958; Sindermann, 1958; Suzuki, Shimizu and Morio, 1958; O'Rourke, 1959; Sindermann and Mairs, 1959; O'Rourke, 1960; Ridgway and Klontz, 1960). While most of this recent interest has been stimulated by application of red cell techniques to the identification of subpopulations, use of the varied methods available to serology can provide information on any taxonomic level.

Comparative serological studies of serum proteins began over half a century ago with the work of Nutall (1901) and have been pursued vigorously in recent decades by Boyden (1926, 1942, 1943, 1954), Gemeroy (1943), Leone (1949, 1950, 1954), Leone and Pryor (1954), Leone and Wiens (1956) and others. This work has demonstrated the utility of serum proteins in systematic studies, especially of higher taxonomic groups. Quantitative examination of the serological relationships of several species within a genus or family have been made (Stallcup, 1954; Leone and Wiens, 1955). Clear distinctions of species are possible with highly specific antisera; even species hybrids, such as the hinny and mule, may be distinguished from parental species (Boyden, 1942, 1953). Comparative serological studies using erythrocyte antigens have been most useful in distinguishing geographic groups, strains, subpopulations or races within a species (Owen, Stormont and Irwin, 1947; Moody, 1948; Ridgway and Klontz, 1960; and others). Irwin, Cole and Gordon (1936) and Irwin (1938, 1955) determined serological relationships of avian species and species hybrids using cellular antigens.

The present investigation was undertaken in 1958 to clarify the natural relationships of several clupeoid species from the western North Atlantic, as well as to establish a serological baseline for concurrent studies of intraspecies groups of herring. The following clupeoids were compared: alewife, *Alosa pseudoharengus* (Wilson); blueback herring, *Alosa aestivalis* (Mitchill); American shad, *Alosa sapidissima* (Wilson); Atlantic herring, *Clupea harengus harengus* Linnaeus; Atlantic menhaden, *Brevoortia tyrannus* (Latrobe). Four methods were used: (1) precipitin tests read photoelectrically with a Libby photronreflectometer; (2) precipitin tests visualized by agar diffusion; (3) erythrocyte agglutination with absorbed antisera; and (4) paper electrophoresis. This composite approach permitted scrutiny of species relationships from several serological viewpoints.

Materials and Methods

Blood samples were collected from fish caught in commercial trap nets on the New Jersey coast. The sampling was done in the spring, summer and fall of 1958,

[1] Present address: Fish Control Laboratory, La Crosse, Wisconsin.

spring and winter of 1959, and spring of 1960, using the cardiac puncture method described by Perkins (1957). Samples were held at approximately 4° C. until clotting had occurred; the serum was then decanted and either used immediately or stored at −20° C. Cells for antiserum absorptions and agglutination tests were washed from the clots remaining.

1. Photronreflectometer

Since photronreflectometer determinations and agar diffusion are but different methods of visualizing precipitin reactions, the same rabbit antisera could be used for both. Pooled serum samples constituted the antigens for each species of fish tested. Antisera were prepared in rabbits by six or twelve subcutaneous injections of .5 ml. of antigen given on alternate days. Only a single series of antigen injections was given, to obtain as specific antisera as possible, in accord with findings of Wolfe and Dilks (1946) and Leone (1952). This resulted in antisera of somewhat lower titer, which are subject to relatively larger experimental error when tested than are strongly reacting antisera. However, an attempt was made to reduce any such error by repeating tests of each serum-antiserum combination. Photronreflectometer determinations were made with a 20-minute reaction time, which provides greatest separation of antigens from closely-related species (Leone 1950). Turbidimetric precipitin tests were carried out following the methods outlined by Boyden and DeFalco (1943) and Leone (1949). Constant amounts of antiserum were added to doubling dilutions of antigen, 1:250 to 1:625,000. This provided a titration curve with a peak at optimal proportions of the reactants, and with slopes to extreme antigen excess on one side and extreme antibody excess on the other. Whole curve comparisons of reactions were made, using summated turbidities of all the antigen concentrations tested, this summation being proportional to the area subtended by the curve. The homologous reaction was designated as 100%, so the summated turbidity (relative area) of the curve obtained with each heterologous antigen could thus be related to the homologous reaction as a per cent. Since five species were compared, five curves were obtained for each antiserum.

2. Agar diffusion

The agar diffusion technique was essentially the method originally described by Ouchterlony in 1948, and since used by Bjorklund (1952a, 1952b), Leone, Leonard and Pryor (1955), Casman (1958), Wilson (1958), Morrill (1959), Ridgway (personal correspondence) and others. A medium of 1.5% Difco agar and .72% sodium chloride was prepared and a basal layer of 8 ml. poured into a flat-bottomed Petri dish. After the basal layer hardened, cylinders of glass tubing (1 cm. in length, 4 mm. inside diameter) were placed in position, one in the center for antiserum and others set equidistantly around it, for the sera of the five species. The center of each serum receptacle was 1.5 cm. from the center of the antiserum receptacle. After a second 8-ml. layer of medium had been added and hardened, undiluted antisera and pooled sera were placed in the cylinders in amounts of .25 ml. per receptacle. The plates were then sealed with masking tape, labelled, and incubated at a temperature of 35.5° C. for seven days. Reading of the precipitate patterns was visual, although photographs were made of some plates.

3. Erythrocyte agglutination

Three rabbits were immunized with pooled washed cells of each species tested. Six or nine .5-ml. doses were injected subcutaneously on alternate days. Trial bleedings were made 10 days after the last injection and if the antiserum titer was adequate, food was withheld and the animals bled terminally on the following day. Antisera were frozen in 3-ml. aliquots at −20° C. until absorptions and cell agglutination tests were carried out.

Cells of each clupeoid species used for antiserum absorptions were washed three times in 1.5% saline, and approximately equal amounts of erythrocytes from all individuals in the sample were pooled. Antiserum was diluted 1:4 and added to pooled cells in proportions of four parts antiserum to one part cells. After 10 minutes of absorption the cell-antiserum suspension was centrifuged and the absorbed antiserum tested against a previously removed aliquot of cells used for absorption. Absorptions of antisera to all five species with each of the five erythrocyte pools were done simultaneously, and one absorption was usually sufficient to remove all antibodies reactive with the absorbing cells. Agglutination tests were then carried out, using all possible combinations of absorbed antisera and cells from each of the five species. Each agglutination test used .2 ml. absorbed antiserum and .05 ml. of 4% cell suspension. Readings were taken after 15 minutes of incubation at room temperature and 30 seconds' centrifugation.

4. Electrophoresis

Paper electrophoresis was carried out with a Spinco Model R system for six hours at 15 milliamperes, using a veronal buffer of pH 8.6 and ionic strength of 0.05. With each run, a sample of human serum was used as a control. Each sample consisted of .01 ml. of serum. Representative curves were obtained by use of a photoelectric densitometer.

Results

1. Photronreflectometer

The results of serological comparisons of five species of clupeoids, using the photronreflectometer with one series of highly specific antisera, are summarized in Figure 1. Comparative values for reactions of each antiserum are in vertical columns beside each curve. Heterologous antigen reactions are presented in per cent of the homologous reaction. Reciprocal relationships show good agreement in most cases, especially in view of the fact that each antiserum must be considered as a separate entity as far as its specificity (discriminating capacity) is concerned.

The serological relationships of the five species presented in Figure 1 are obviously not linear, but may be expressed satisfactorily in a three-dimensional graph (Fig. 2) based on "serological distances" calculated by subtracting the per cent heterologous reaction from 100 (Boyden, 1926). This converts the relative correspondence of that particular antigen to a "relative distance" value. With this method antigens closely related to the homologous antigen will be relatively close to it, while those antigens of increasing dissimilarity will be increasingly far from the homologous. For example, in Figure 1 the serum proteins of the alewife gave a reaction that was 90% of the homologous reaction between blueback serum and

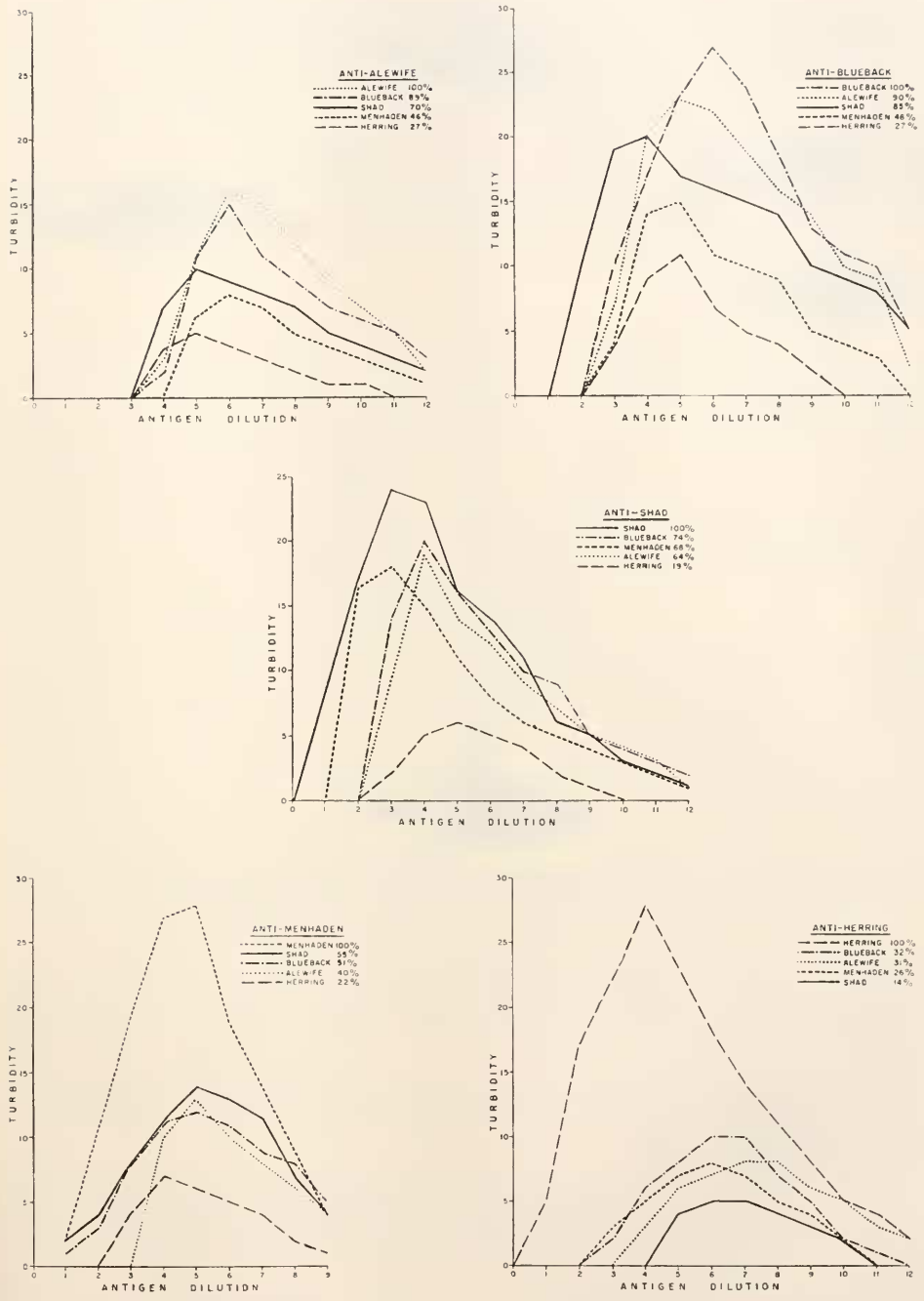

FIGURE 1. Precipitin curves derived from reactions of serum antigens of five clupeoid species with antisera prepared to each species.

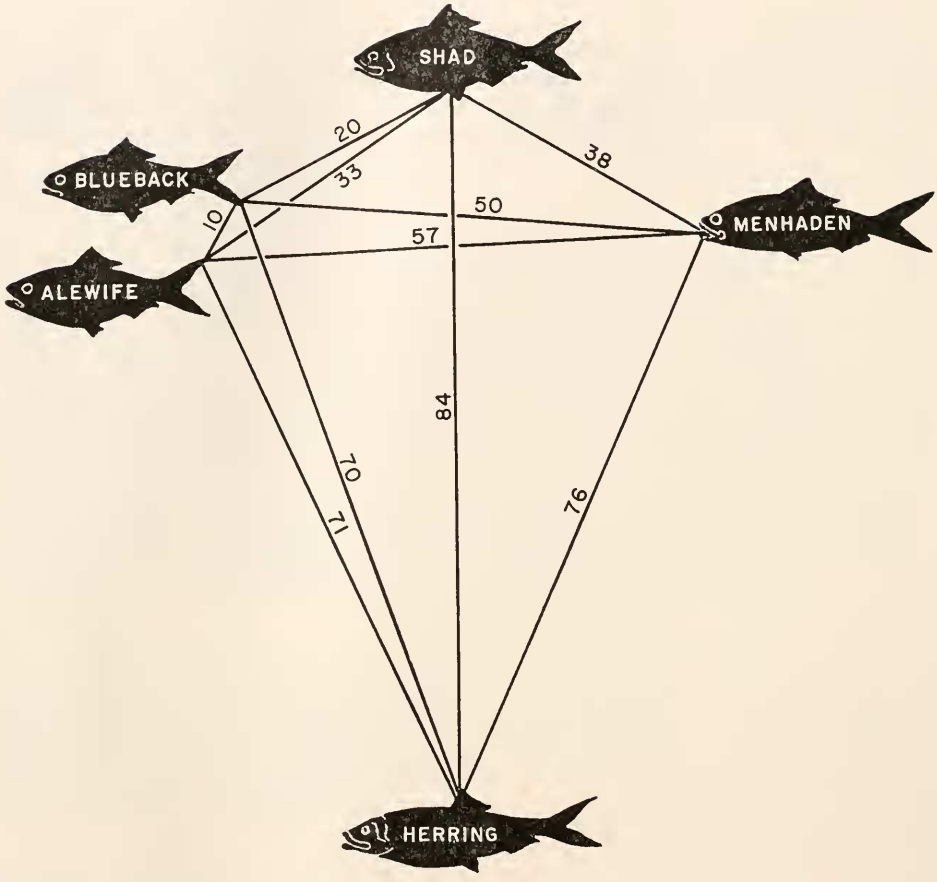

FIGURE 2. Three-dimensional representation of the present relationships of five clupeoid species, as determined from precipitin tests read with the photronreflectometer.

anti-blueback serum. The relative serological distance between blueback and alewife with this antiserum is thus 10. However, the reaction of herring serum proteins with the anti-blueback serum was only 27% of the homologous, so the relative serological distance between blueback and herring with this antiserum is 73. All relative distance values between any two species were averaged in preparation of Figure 2, and each species locus was determined by average distance values from each of the other species.

The data indicate that blueback and alewife have a high degree of serological correspondence; that shad are closer to the blueback-alewife complex than to either menhaden or herring; and that menhaden and herring are remote from all others, with herring consistently at the greatest serological distance from the other four species.

The numerical relationship values obtained are not fixed, but reflect results obtained with the particular antisera used. The relative positions of species with re-

spect to one another should, however, remain relatively constant. Boyden, DeFalco and Gemeroy (1951) and Boyden (1953) have demonstrated that despite variations in specificity and systematic range of several antisera of the same kind, a consistent placement series will emerge for the species tested. This was demonstrated in the present work by a second series of antisera with lower specificity obtained by deliberately prolonged injections extending over a one-month period. Such antisera were in most instances less specific, but no variations in relative placement of the five species occurred. Serological distances separating heterologous and homologous species were reduced in most cases, but relative positions and placements were similar.

2. Agar diffusion

Clear patterns of precipitate were obtained with each of the antisera used. Although minor differences were noticed in the discriminating capacity of different rabbits immunized against the same species, the reproducibility was high with respect to number and position of precipitate bands in the gel. Diagrammatic sketches of typical patterns appear in Figure 3.

Since results of agar plate tests are very difficult to quantitate, they are not as useful for the determination of exact serological distances between species as the photronreflectometer. However, since closely related species display similar reactions and share some precipitate bands in reactions of identity, it is possible to get a good general idea of the relationships involved.

Alewife and blueback sera showed very similar reciprocal reactions. Shad serum reacted strongly to antisera prepared against alewives and bluebacks and shared some precipitate bands with these species, but the reciprocal reactions were somewhat weaker; thus, in the overall picture shad must be placed slightly farther from alewives and bluebacks than these two species are from one another. Menhaden and herring appear to stand somewhat apart from the alewife-blueback-shad complex, but the reactions of menhaden showed a closer relationship to that group than did those of herring. Apparently, menhaden and herring have very little taxonomic affinity, for antisera prepared against either of these species evoked only the faintest traces of precipitate from sera of the other, and these trace reactions were always of the type representing complete non-identity of antigens.

The results of the agar diffusion tests substantiate the more precise picture of relationships shown by the photronreflectometer. The positions of the species are in the same orientation as those deduced from the turbidimetric measurements.

3. Erythrocyte agglutinations

Tests were made with reagents obtained by absorbing each antiserum with cells of each of the five species. The reactions (Table I) represent composites of three separate tests, each using different samples of fish blood and a different antiserum. Results are recorded conventionally in descending order from (++++), representing complete agglutination, to (−) representing no agglutination.

The reduction in strength of agglutinations after absorptions indicates that all antisera contained substantial amounts of cross-reactive antibodies. Alewife and blueback cells shared many antigens, so that absorptions with either gave very similar but not identical reactions. Shad and menhaden shared some antigens, so

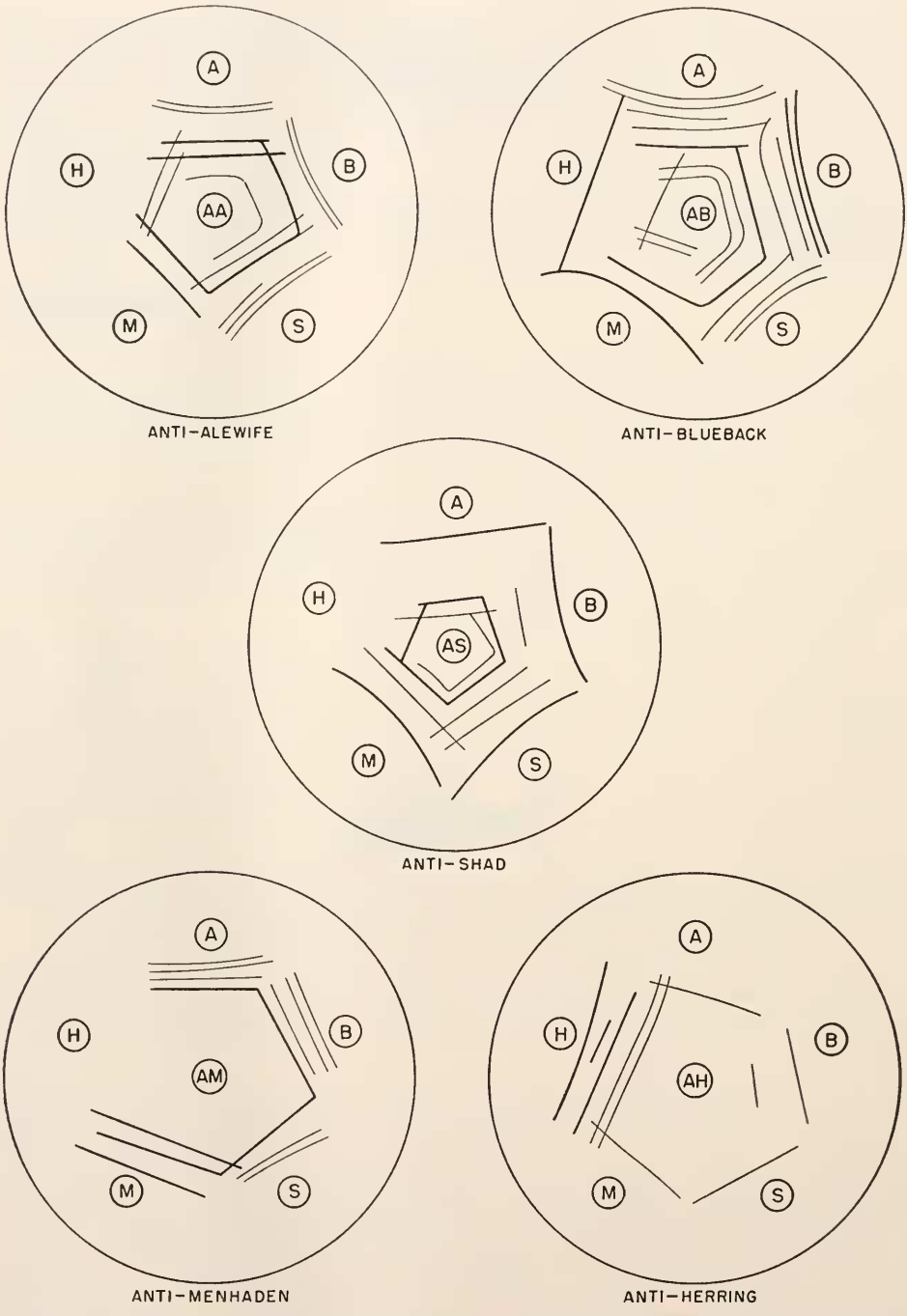

FIGURE 3. Diagrams of reactions in representative agar diffusion plates for each of the five clupeoid species. A = alewife, B = blueback, H = herring, M = menhaden, S = shad.

TABLE I

Reactions of erythrocytes of five clupeoid species with absorbed antisera

Antiserum	Cells used in absorptions	Agglutination reactions				
		Alewife	Blueback	Herring	Menhaden	Shad
Alewife	Alewife	−	−	−	−	−
	Blueback	+	−	+	−	−
	Herring	+++	++	−	−	+
	Menhaden	+++	++	++	−	−
	Shad	++	+	++	−	−
	Unabsorbed	+++	++	+++	+	++
Blueback	Alewife	−	−	+	−	−
	Blueback	−	−	−	−	−
	Herring	++	+	−	−	−
	Menhaden	++	+	+++	−	−
	Shad	++	+	++	−	−
	Unabsorbed	++++	+++	++++	+	++
Herring	Alewife	−	−	+	−	−
	Blueback	+	−	++	−	−
	Herring	−	−	−	−	−
	Menhaden	+	+	+++	−	−
	Shad	−	−	++	−	−
	Unabsorbed	++++	++	+++	+	++
Menhaden	Alewife	−	−	++	+	+
	Blueback	+	−	++	+	+
	Herring	+	+	−	+	+
	Menhaden	−	−	−	−	−
	Shad	+	+	++	+	−
	Unabsorbed	+++	++	+++	++	++
Shad	Alewife	−	−	+++	+	++
	Blueback	+	−	+++	++	++
	Herring	+	−	−	++	++
	Menhaden	+++	++	+++	−	+
	Shad	−	−	−	−	−
	Unabsorbed	+++	++	+++	+++	+++

that absorptions gave a somewhat similar pattern of reactions, although the relationship was by no means as close as that of alewife and blueback.

4. Electrophoresis

Electrophoretic analyses were made with both pooled and individual sera. Individual sera appeared in all cases to give better definition of components, although they conformed to the general pattern of the pools. Considerable intraspecific variation in patterns was evident, but a generalization of each species pattern was possible. The curves derived from these generalized patterns are depicted in Figure 4, as determined by densitometer readings; they are, however, not intended to illustrate absolute species specificity. The numbers designating each peak are

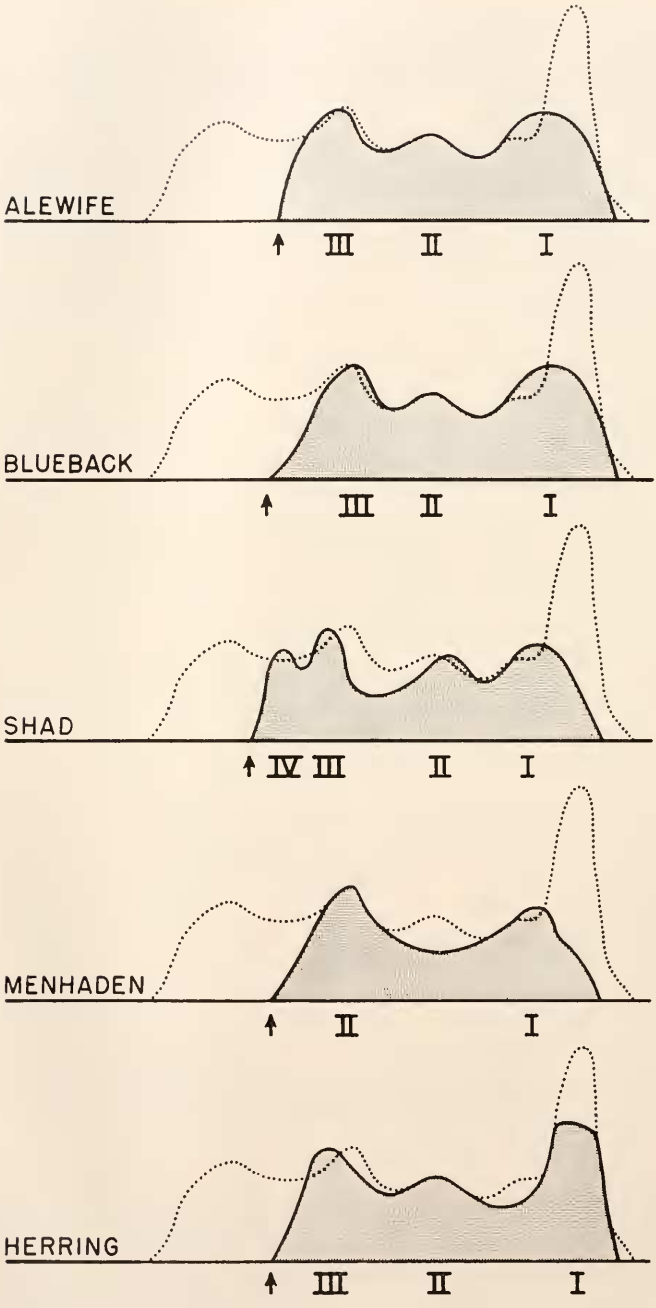

FIGURE 4. Representative paper electrophoresis diagrams for five clupeoid species as determined by densitometer readings. Dotted line represents human serum used as control.

used for ease in description and do not denote identity of components between species.

The alewife and blueback sera displayed very similar electrophoretic patterns, each with three major fractions. The main difference between the two species was found in the least mobile fraction; in alewives this fraction was less mobile than human beta globulin, whereas in bluebacks it migrated further than human beta globulin. Both species had a weak fraction of the same mobility as human alpha-2 globulin, and a fraction of slightly less mobility than human albumin. In some specimens of alewife, a weak fourth fraction appeared, intermediate in mobility between the human alpha-2 and beta globulin fractions.

The shad samples tested displayed a pattern of four fractions. The least mobile of these did not migrate as far from the point of application as human beta globulin. A strong fraction with slightly more mobility was present in all specimens, as were moderate fractions with slightly more mobility, respectively, than human alpha-2 and alpha-1 globulins.

The pattern shown by the menhaden sampled had two fairly strong fractions. One of these was identical in mobility to human beta globulin, and one was intermediate between human alpha-1 globulin and albumin.

Three moderately strong fractions were obvious in the patterns of the sea herring tested. The least mobile migrated a shorter distance than human beta globulin, while the others travelled the same distances, respectively, as human alpha-2 globulin and albumin. In some fish of this species a fourth fraction appeared which was intermediate between human alpha-1 globulin and albumin.

Discussion

1. Photronreflectometer

The clear differentiation of five clupeoid species with this precipitin technique read photoelectrically offers tempting possibilities for future studies. It would be interesting, with the background of the present data, to compare other morphologically similar but geographically isolated clupeoid species. Suspected hybrids, such as those between *Alosa pseudoharengus* and *A. aestivalis* might also be examined serologically. Comparison of populations or "races" of cosmopolitan species, such as *Clupea harengus,* with serum techniques might also prove instructive.

The limitations of such studies must be kept in mind. Results of serological examinations would not be considered as the sole criterion for taxonomic conclusions, but should be evaluated together with morphological and other data to provide as broad a base as possible for such conclusions. Information about the possible influences of environmental and physiological changes on serological reactions should be obtained. It should also be emphasized that results will indicate present serological relationships, but will give only indirect information about the evolutionary history of the species concerned. Despite such limitations, serum techniques offer possibilities in fisheries research that should be explored as vigorously as other techniques for the understanding of natural relationships.

2. Agar diffusion

Further experimentation should be conducted with agar diffusion to determine its usefulness in studies on fish populations. Absorption of antisera to remove spe-

cies antibodies would probably be a virtual necessity for work at the intraspecific level.

The advantage of this type of test obviously lies in the fact that the total precipitate is resolved into its component antigen-antibody reactions, which are subject to direct visual observation. While this permits a high degree of qualitative differentiation and gives a good idea of general species relationship, it is very difficult to measure exactly the total amount of precipitate formed. For this reason, it is best to leave the final calculation of relative serological distances between species to turbidimetric methods, keeping in mind the fact that rabbits or other experimental animals are not the exclusive and final arbiters of natural relationships.

3. Erythrocyte agglutination

The use of pooled cells to absorb antisera to all five clupeoids, and the subsequent testing of such pools with reagents obtained by absorptions, have provided a criterion for determining the relationships of five clupeoid species. It would be instructive to apply this test to additional clupeoid species in other regions.

Another logical extension of this work would be determination of the distribution of similar or identical antigens in individual blood samples of the five species. Individual differences have been noted in the present work for all five species, but, except for herring, blood group systems have not been proposed. The use of pooled cells masks individual differences, and it is obviously less precise than a study of discrete antigens with specific reagents. To test the utility of the single antigen approach, individual samples of all species were tested with the reagents used in routine examination for the C blood group antigen of herring (Sindermann and Mairs, 1959). Positive reactions were obtained with most alewives, half of the bluebacks, a few shad, and no menhaden, indicating the existence of the same or at least a closely related antigen in species other than herring. Similar tests with other specific reagents could create a mosaic of reactions that would provide a clearer picture of the affinities disclosed by the present study. The utility of such an approach has already been demonstrated with certain mammalian species by Stormont and Suzuki (1958).

4. Electrophoresis

Although it was possible to construct electrophoretic patterns characteristic of the five species of clupeoids, the intraspecific variations encountered in this study and in an electrophoretic examination of herring populations (Mairs and Sindermann, 1960) indicate that great caution should be employed in the establishment of specific patterns for teleostean species. It has been shown that the component fractions of serum display variability in both quantity and electrophoretic mobility depending on such factors as disease, age, sex and starvation (Moore, 1945; Dessauer and Fox, 1956; Drilhon *et al.*, 1956; Sindermann and Mairs, 1958). This variability could lead to significant overlap and confusion of patterns, especially among closely related species. In many cases, it is doubtless possible to characterize a high proportion of the electrophoretic patterns in a sample, but proposal of a species-specific pattern would have to follow testing of a large number of individuals, with close reference to maturity, disease, and any other physiological or environmental factor known or likely to affect electrophoretic characteristics. Only in

this way could a species "norm," if such exists, be properly defined and an insight gained on the true significance of the variations encountered.

The present study indicates that, while paper electrophoresis may have some general usefulness in fish taxonomy, immunological techniques are preferable for precise differentiation and attempts at determining natural relationships.

Summary and Conclusions

1. An investigation of the serological relationships of five species of clupeoid fishes was made by four methods: (a) photronreflectometer, (b) agar diffusion, (c) erythrocyte agglutination with absorbed antisera, and (d) paper electrophoresis.

2. Agar diffusion enabled qualitative differentiation of the species tested, while the photronreflectometer provided a quantitative measure of relative serological distances between species; results from the two methods were in good agreement.

3. On the basis of results obtained by the photronreflectometer and agar diffusion methods, the following species relationships are indicated: alewives and bluebacks are very closely related; shad lie quite close to alewives and bluebacks, but farther from them than alewives and bluebacks are from one another; menhaden and herring are further removed from the alewife-blueback-shad complex, with menhaden closer to it than herring; herring are comparatively remote from the other four species.

4. Generalized electrophoretic patterns were found for each clupeoid species. However, because of intraspecies variability, paper electrophoresis does not seem to be as useful a procedure for determining relationships of fishes as immunological methods. Species-specific patterns should be proposed only after large numbers of individuals representing both sexes have been sampled under different physiological conditions.

5. Absorptions of rabbit antisera with pooled erythrocytes of each of the five clupeoid species indicated that alewife and blueback were antigenically very similar; menhaden and shad were antigenically somewhat similar, although not as close as alewife and blueback.

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